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PRINCIPAL INVESTIGATOR: Yasmine Kanaan, Ph.D.

CONTRACTING ORGANIZATION: Howard University
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Yasmine Kanaan, Ph.D.

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**14. ABSTRACT**
Genes may play a strong role in prostate cancer etiology but epidemiological studies suggest that prostate cancer risk is largely determined by gene and environmental interactions. In order to explore the effects of UV exposure, serum Vitamin D, and skin color on prostate cancer risk in African American men. Ninety affected AA men with histologically diagnosed adenocarcinoma of the prostate; PSA of > 2.5 ng/ml and a positive DRE were recruited under the direction of Dr. Mireku-Boateng from the division of Urology at the Howard University Hospital and forty age and ethnicity matched controls have been recruited through the monthly free screenings program at the Howard University Cancer Center. For each prostate cancer patient and matched control we have collected information on personal and family history, and blood samples for candidate gene testing. In order to measure the intake of dietary Vitamin D each subject completed the standardized food frequency questionnaire and the serum circulating levels of 25-OH Vitamin D have been measured by Enzyme Immunoassay for all participants. To elucidate their exposure to UV from childhood until current the UV exposure questionnaire has been completed. In addition their constitutive skin color (M-index) measured has been done by using the dermaspectrophotometer.

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Ultraviolet Radiation (Uv), Skin Color, Vitamin D Metabolism,Single Nucleotide Polymorphism, African Americans

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## INTRODUCTION

There are striking differences in the age-adjusted incidence of prostate cancer between different racial groups and between different geographic regions of the world. African American men have the highest incidence of prostate cancer compared to other ethnic groups. This cohort also appears to be present more commonly at an advanced stage with aggressive histology and increased cancer-related mortality. Therefore, there is a critical need to explore the etiologic pathways that contribute to this disparity.

There is accumulating evidence that vitamin D may play an important determinant of occurrence and progression of prostate cancer. Because the prostate cancer mortality rate increases significantly as the availability of UV radiation exposure decreases, and the synthesis of vitamin D depends on UV radiation, it was hypothesized that vitamin D deficiency is a risk factor for prostate cancer (1). Laboratory studies revealed that vitamin D and vitamin D analogues have anti-proliferative and differentiation effects on human prostatic cancer cells *in vitro* (2-10). Clinically, it was claimed that oral administration of 1, 25- dihydroxyvitamin D3, an active form of vitamin D, may delay the recurrence of prostate cancer after primary therapy (11). These documents suggest that vitamin D had a protective effect on prostate cancer

Genes may play a strong role in prostate cancer etiology but epidemiological studies suggest that prostate cancer risk is largely determined by gene and environmental interactions. Increased attention should be placed on research in the African American population on environmental factors such as UV exposure (latitude), lifestyle and diet and their possible interactions with genetic loci (12-14) .

**The goal of this project is** to explore the effects of UV exposure, serum Vitamin D, and skin color on prostate cancer risk in a large case-control study of African American men aged $\geq$ 40 years from the Washington, DC area. **Our specific aims are** to (1) recruit 76 prostate cancer cases and 152 age and ethnicity matched controls; (2) assess UV exposure in African Americans prostate cancer patients and matched controls (3) measure modifying factors of UV exposure (skin color, serum 25-OH Vitamin D, and genes involved in Vitamin D metabolism (4) assessment of variation for genes involved in vitamin D metabolism; and (5) determine if UV exposure and modifying factors act alone or interact to affect prostate cancer risk in African Americans.

## BODY

### Task 1: Start-up phase and clinical database development (1-5 months)

- Develop detailed protocol manual for recruitment and database entry.
- Start advertisements and recruitment process.

**Start-up Phase:** After the grant activation (September, 2005) we started hiring staff (Dr. Desta Beyene and graduate students); familiarizing faculty and staff with the UV exposure questionnaire (UVQ), the standardized food frequency Questionnaire (FFQ); and the training for using the DermaSpectrophotometer machine for measuring the constitutive skin color which was done under the supervision of Dr. Halder. Dr. Kanaan and Dr. Lucille-Adams Campbell met with the research team, discussed the role of each member in the project, and will continue the meetings in regularly base.

Ethics training: The students had completed the Howard University Collaborative IRB Training Initiative (CITI) Course and Human Participants Protection Education for Research Teams (online course, sponsored by the National Institute of Health) Course.

**Clinical database development:** The database has been developed by Dr. Makambi, all the questionnaires were barcode labeled and entry of data will be checked by the statistician Dr. Makambi for quality control. Validation of the data will take place both during and after data entry, with attention to the following checks: Variable Type, Range, List of Possible Values, Internal Logical, Data completeness and Duplicate Record Checks. The data will be inspected to prevent the entry of duplicate records into the database. Corrections to the data set will be made by a) reselecting the appropriate form, b) querying the data for the record, and c) updating the incorrect information. Estimates of the rate of entry error and the identification of any systematic data entry errors will be found by comparing printouts of entered data to the data encoded on the individual's questionnaire(s). These manual audits may be conducted on a periodic basis. Daily backups will be conducted to protect against accidental corruption or deletion of essential data.

**Recruitment Protocol and Data Collection**

We started advertisement process for the project after we received Howard University Institutional Review Board (IRB) and Army Surgeon General's Human Subjects Research Review Board (HSRRB) approval in July, 2005 and recruitment process after the grant activation on September, 2005.

The selection of prospective prostate cancer cases and healthy controls began with the identification of potential participants from a variety of resources available to Dr. Augustine Mireku-Boateng, such as patient files, tumor registry, patient databases, and Cancer Center prostate cancer screenings. Howard University Hospital (HUH) has historically served the African American community in the Washington, DC area. To date we have recruited 90 affected African American men with histologically diagnosed adenocarcinoma of the prostate; Prostate specific antigen (PSA) of $\geq$ 2.5 ng/ml and a positive digital rectal examinations (DRE) under the direction of Dr. Mireku-Boateng, from the division of Urology at the Howard University Hospital and/or from ongoing HUH/HUCC free prostate cancer screening programs at the Howard University Cancer Center (HUCC). Forrty age and ethnicity matched controls healthy unaffected regularly screened volunteers with PSA levels < 2.0 ng/ml, normal DREs and with no history of prostate cancer among first-degree relatives were also recruited from the HUH urology division and from ongoing HUH/HUCC free prostate cancer screening programs at the Howard University Cancer Center. All case and controls are not related.

All participants from the study have consented by the Recruiter and filled out the questionnaire on demographic and medical history. Each individual was assigned a unique identification number. The numbering system allowed us to track individual samples without the need of personal identifiers (i.e., names and addresses). The numbers were assigned sequentially; Blood were collected from all participants (3 yellow top Vacutainer 6ml tubes for lymphocytes and DNA extractions and 1 red top Vacutainer 6ml tubes for blood chemistry) by certified phlebotomists.

For each prostate cancer patient and control we obtained information on personal and family history, blood samples for candidate gene testing. Each subject answered questions from the UV exposure questionnaire (UVQ) and the standardized food frequency Questionnaire (FFQ) in addition to having their constitutive skin color (M-index measuring using the Dermaspectrophotometer).

**Demographic and Medical History:** Prior to the in-person interviews, all subjects signed informed consent. For each prostate cancer patient and matched control we have collected information on personal and family history. Personal history includes ethnicity, alcohol and tobacco intake, occupation exposures, height and weight, medical history and physical activity, and blood samples for candidate gene testing.

We also collected information about household income, home ownership, number of children, and employment of the participant, as well as the highest level of education completed. Collected variables will allow us and other investigators to assess the association between these variables and clinical features.

For the prostate cancer cases, age of onset (mean 68.5 years, range 47-84), Gleason score (range 4-9) and PSA level (range 8.2-80 ng/ml) were obtained from the cancer registry. Most men are resident in Washington area and the remainder from surrounding Metropolitan areas.

All subjects signed informed consent, and the Howard University Research Center Institutional Review Board approved study forms and procedures. Since there is no further contact with the subjects, there are minimal physical, psychological, social, or legal risks involved in this study.

Confidentiality of the participants has been and will continue to be fully protected. No personal identifiers were recorded and transmitted with the blood samples and clinical data. All personal information on participants is kept separate in a locked cabinet by the Principal Investigator.

**Assessment of UVR exposure:** We have established the protocol for calculating each subject's cumulative sunlight exposure. Each subject answered questions from the UV exposure questionnaire (UVQ). This questionnaire is designed to elucidate their exposure to UV from childhood until current. Subjects were asked to assess such exposures during age categories; 0-5 years, 6-11 years, 12-17 years, 18-29 years, 30-39 years and 40 years-to age at diagnosis. These data were combined to give total UVR exposure in hr/year.

In the protocol used in this study, each subject's cumulative sunlight exposure will be assessed by a combination of his history of occupational and non-occupational sunlight exposure. All professions were listed and the time periods during which these profession were performed, were recorded.

In addition to these questions the patients were also asked the following questions, which were not included in the article in the New England Journal of Medicine. They were asked to indicate their sunlight exposure during specified age periods as "low", "moderate", and "high". Age periods were chosen in such a way that they were easily recognizable, namely ranging from 0 - 5 years: baby time, toddler time and infant class, 6 - 11 years: "lower" school period, 12 - 17 years: "middle" school period, 18 - 29: University, first profession etc.

**Food Frequency Questionnaire:** Additionally, each subject completed the standardized food frequency questionnaire (FFQ) for dietary assessments. The FFQ is an appropriate epidemiologic method for dietary assessment and is designed to obtain qualitative and descriptive information about usual food consumption patterns. Specifically, the 98.2-item Block Brief 2000 questionnaire will be determined. The Block questionnaire was developed using previously described methods (15), with a food list designed to cover greater than 90% of the average intakes of over 30 nutrients in Whites, African-Americans, and Hispanic Americans. The questionnaire is self administered and shows how often food is consumed as number of times per day, week, months or year. The usual portion size is reflected as small, medium, or large with a picture representation of these sizes. The Block FFQ has been validated and used to

assess dietary intake in an African American population (16). The completed FFQs will be sent to the Block Dietary Data Systems in Berkely, CA for analysis.

**Assessment of Skin Color:** Measurement of skin pigmentation was carried out using the computerized narrow-band reflectometer called the Derma Spectrophotometer (Minolta Chromameter, Courage and Khazaka Mercameter). Using two wavelengths, the instrument records the reflectance of light emitted on the skin. The results are expressed in terms of erthema (E) and melanin (M) indices (0 to 100%). The inner arm is used to measure constitutive skin pigmentation. Measurements on the forehead and back of hand (facultative skin pigmentation) were taken for the exposed skin area. Three separate measurements of E and M are taken at all three sites and the average M value used in the analysis. The difference between constitutive (inner arm M) and facultative (forehead M) has been proposed as a quantitative index of sun exposure that is related to cumulative lifetime sun exposure (tanning potential).

## Task 2: Data collection (Months 3-24).

- Extraction of genomic DNA from blood samples.
- Run serum 25-OH Vitamin D assays.
- Enter clinical data in database.

**DNA Extraction:** After the interview, blood was drawn from consenting subjects. The lymphocytes were separated from the blood of patients and controls by using Lymphoprep solution (Axis-Shield POCAS, Oslo, Norway). The solution contains 9.1% (w/v) sodium diatrizoate and 5.7% (w/v) polysaccharide. It is a one-step centrifugal technique for isolation of lymphocytes. Genomic DNA was isolated from whole blood using the QIAamp DNA Blood Maxi isolation kit as described by the manufacturer (QIAGEN Inc.). The procedure involved cell lysis, proteinase K-treatment, protein precipitation and DNA precipitation. The DNA concentration was determined by the Nano-Drop (ND-1000). The collected Blood and DNA from Subjects is stored in a locked freezer which is located in a secured area at Howard University Cancer Center and an identification code were used on blood samples.

**Serum 25-OH Vitamin D assays:** We have established and standardized the conditions in our laboratory the assay for the quantitative determination of the 25-OH Vitamin D in serum by using the Enzyme-based-Protein-Binding-Assay from ALPCO (Windham, NH). Photometric measurements of the standard concentrations (0, 6.4, 16, 40, 100 and 200 nmol/L) were used to establish a standard curve using a four-parameter model. The equation used was $Y= (a-d)/(1+(x/c)^{b}) +d$, where y= absorbance reading @450nm; a= 1.20958, b=0.71301, c=19.72576, d=0.06209 and x= concentration in nmol/L. The actual Vitamin D concentration will be calculated based on the relationship of 1ng/ml = 2.5 nmol/L

This dose response curve of the absorbance unit vs. concentration is generated using the results obtained from the calibrators. Concentrations of 25-OH Vitamin D, present in the subjects' samples, will be determined directly from this curve.

**Denaturing High-Performance Liquid Chromatography (DHPLC):**

DHPLC analysis is a chromatographic mutation analysis method that relies on the formation and separation of double-stranded DNA fragments that contain mismatched pairs from a pool of PCR amplified DNA fragments known as heteroduplex DNA. DHPLC is based upon heteroduplex detection and the heteroduplex profiles are identified by visual inspection of the chromatograms on the basis of the appearance of additional earlier eluting peaks. Corresponding homozygous profiles show only one peak. Analysis is carried out on an automated DHPLC instrumentation equipped with a DNASep column (Transgenomic Inc., San Jose, CA). Samples will be separated (flow rate 0.9 ml/min) through a linear acetonitrile gradient (Fisher, Chicago) and detected by online ultraviolet (UV) absorbance monitoring at 254 nm.

We have already standardized the polymerase chain reaction (PCR) and DHPLC conditions for analyzing the vitamin D receptor (VDR) gene. Polymerase chain reactions (PCR) of VDR exons and intron-exon boundaries as well as the promoter region were performed in a 25 ul volume (containing 40 ng of genomic DNA as a template, 0.4 uM each of exon-specific forward and reverse primers , 1X Gold Buffer, 0.2 mM dNTP mix; 2 mM $MgCl_2$ and 1 U AmpliTaq Gold polymerase. PCR was performed in an AmpGene 9700 thermal cycler (Perkin-Elmer 600, Foster City, CA) as follows: initial denaturation at 95 $^0$C for 3 min to activate the enzyme; 5 cycles of 30 sec at 95 $^0$C, 40 sec annealing at 65 $^0$C (decrement of 2 $^0$C per cycle), and 1 min extension at 72 $^0$C followed by 45 cycles of 30 sec at 95 $^0$C, 40 sec at 55 $^0$C, and 1 min at 72 $^0$C; and final extension at 72 $^0$C for 7 min. The PCR annealing temperature for each exon was determined using the Mac Vector software. Crude PCR products were checked by agarose gel electrophoresis before DHPLC analysis, to make sure that no additional bands occurred that could lead to artificial heteroduplex conformation. The PCR products were subjected to an additional 10 minutes at 95 $^0$C denaturing step and then left at room temperature for 10 minutes for reannealing prior to analysis. The start-and end-points of the gradient were adjusted according to the size of the PCR products using an algorithm provided by the WAVE maker system control software. The temperature required for the optimum resolution of heteroduplex molecules is determined by use of the DHPLC melting algorithm and pre-testing of several temperatures. The samples that show heteroduplex peaks will be sequenced in both directions on an ABI 377 automated sequencer using the fluorescent labeled Big-dye terminator cycle sequencing kit (ABI) or ET terminator cycle sequencing kit (Amersham).

**Entering clinical data in database:** The data were entered into template forms written in Microsoft ACCESS. Each site is kept in duplicate records of all data sent to the coordinating center. The study coordinator monitored the transfer of data and blood specimens in addition to hand checking hard copies before entering the data into the database. All data were entered using the double-keying method. The PI worked with Dr. Lucille-Adams Campbell on aspects of the study dealing with the database and data management.

**Preliminary Data:** We have begun building a comprehensive data resource to explore the interactions of vitamin D levels, UV exposure, genes and diet in African American men with and without prostate cancer. Statistical analysis for each study aim planned to exploit univarite and multiple logistic regression models. As seen in the table a summary of the collected data we have so far. The mean age among the cases and controls were similar. Generally, there was a higher level of exonic polymorphism for cases as compared to the controls. The samples have been send to Transgenomic Inc. (San Jose, CA) for sequencing. We are in the process of recruitting more control subjects and these preliminary results will further be examined and verified with a larger samples size and more rigorous statistical analyses.

| **Variable** | **Cases** | **Controls** |
|---|---|---|
| Number of samples | 90 | 40 |
| Mean age, years | 68.5 | 58.6 |
| Mean sunlight hours | 24,764.61 | 24,508.47 |
| Mean Quantitative tanning index | 117.05 | 87.1 |
| Mean tanning potential, % | 18.8 | 25.14 |
| PSA (ng/ml) range | 8.2-80 | 0.4-3.5 |
| **Polymorphic/variations patterns, VDR exons** | | |
| Exon 3 | 4 | 1 |
| Exon 4 | 37 | 11 |
| Exon 5 | 12 | 6 |
| Exon 6 | 7 | 4 |
| Exon 7 | 22 | 10 |
| Exon 8 | 22 | 14 |
| Exon 9 | 44 | 16 |

**KEY RESEARCH ACCOMPLISHMENTS:**

- We have recruited 130 African American men (90 prostate cancer patients and 40 age and ethnicity matched controls from healthy unaffected regularly screened volunteers).
- Each subject answered questions from the UV exposure questionnaire (UVQ) and the cumulative sunlight exposure have been calculated for each subject.
- Each subject had answered the standardized food frequency questionnaire (FFQ) for dietary assessments. The completed FFQs will be sent to the Block Dietary Data Systems in Berkley, CA for analysis at the end of the study.
- A new Derma Spectrophotometer has been purchased and is being used to measure skin pigmentation.
- Measurement of skin pigmentation was carried out using the computerized narrow-band reflectometer called the Derma Spectrophotometer. The difference between facultative (forehead M) and constitutive (inner arm M) readings has been proposed as a quantitative index of sun exposure that is related to cumulative lifetime sun exposure (tanning potential).
- Lymphocytes, serum and DNA from collected blood of subjects have been isolated and stored in a locked freezer which is located in a secured area at Howard University Cancer Center and an identification code were used.

- PCR and DHPLC conditions for detecting variants have been established for VDR and CYP genes.
- Sixty six samples have been send to Transgenomic Inc. for sequencing
- All the subjects' obtained information, i.e. demographic, family and medical history have been entered into the database.

**REPORTABLE OUTCOMES**: Research and laboratory technology training:
The training was for graduate students: Altreisha Foster and Douglas White (Microbiology Department), Hilaire Kenguele (Biology Department) and undergraduate student: Vonetta Williams (Chemistry Department), on the use of technology and software for primers designing, lymphocytes isolation and DNA extraction, and ethics training.
**Ethics Training:** The students had completed the Howard University Collaborative IRB Training Initiative (CITI) Course and Human Participants Protection Education for Research Teams (online course, sponsored by the National Institute of Health) Course.

**POTENTIAL PROBLEM:**

- The sequencing of samples for the detected variations has been hampered because of technical problem of the Sequencer at the Howard University Genomic Center. In the mean time samples have been sent to Transgenomic Inc. for sequencing.

- In order to increase our control subjects, Dr. Aaron Jackson along with Dr. Mireku-Boateng is willing to participate in the study and help in recruiting the controls from the division of Urology at the Howard University Hospital and/or from ongoing HUH/HUCC free prostate cancer screening programs at the Howard University Cancer Center (HUCC). In order to enhance the recruitment process for the control subjects, we'll expand the range of PSA level to 3.5 ng/ml and maintain the other criteria: age and ethnicity matched controls healthy unaffected regularly screened volunteers with normal DREs and with no history of prostate cancer among first-degree relatives, and all cases and controls are not related.

- We have established and standardized the conditions in our laboratory the assay for the quantitative determination of the 25-OH Vitamin D in serum by using the Enzyme-based-Protein-Binding-Assay from ALPCO. But the standard curve used for determining the vitamin D concentration in serum showed very low readings with the subjects' sera and will be repeated for accurate results.
  A report by NIH (attachment) "found it difficult to define specific blood levels of markers for vitamin D status that indicate optimal levels for bone health. One reason for this is that current methods, which measure serum-25-hydroxy vitamin D as the marker for vitamin D status, yield highly inconsistent results. As part of its broader vitamin D initiative, Office of Dietary Supplements (NIH) is working with laboratory testing facilities to standardize the quantification of vitamin D status".

**CONCLUSIONS:** Prostate cancer is a complex disease with both genetic and environmental components. Epidemiological data reveal that African American men have the highest incidence and mortality rates for prostate cancer. Despite its high prevalence among African

Americans, very little is known regarding genetic predisposition and environmental influences on prostate cancer. We are particularly intrigued by the interaction of UV exposure and modifiers of vitamin D level in the serum (skin color, genes and diet). This observation leads to the hypothesis that the higher incidence of prostate cancer in elderly men and black men may be related to vitamin D exposure, which is decreased with aging skin and darker skin pigmentation. Our working hypothesis poses that increased incidence of prostate cancer and mortality in African Americans involves a dynamic interplay of environmental factors such as diet and UV exposure in addition to genetic factors, some which directly influence serum vitamin D levels. Our work thus is extremely promising. Once completely analyzed, our data will contribute to the current knowledge on DNA sequence variations. But more importantly, analyses of our populations will allow us to determine the role DNA sequence variations play in prostate carcinogenesis, response to treatment and disease aggressiveness in high risk populations.

**FUTURE DIRECTIONS:**

- Will continue recruiting Subjects from the Washington, DC area either from the urologic practice through Drs. Augustine Mireku-Boateng and Aaron Jackson at Howard University Hospital. Informed consent for genetic research on prostate cancer will be obtained and documented from all volunteers.

- For each prostate cancer patient and matched control we will obtain information on personal and family history, and collect blood samples for candidate gene testing and serum vitamin D level. Personal history includes ethnicity, alcohol and tobacco intake, occupation, and medical history. Each subject will answer questions from the UV exposure questionnaire (UVQ), standardized food frequency questionnaire (FFQ) and their constitutive skin color will be measured using the Derma Spectrophotometer.

- Sequence results will be analyzed and compared to research data previously reported by other studies on VDR polymorphism

- We will build a comprehensive data resource to explore the interactions of Vitamin D levels, UVR exposure, genes and diet in African American men with and without prostate cancer.

- Statistical analysis for each study aim is planned to exploit univariate and multiple logistic regression models.

- Dr. Kanaan and Dr. Lucille-Adams Campbell will continue meeting regularly with the research team to discuss the outcome and the project’s progress.

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